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Report

# Zooplankton May Serve as Transmission Vectors for Viruses Infecting Algal Blooms in the Ocean

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## Summary

**Marine viruses are recognized as a major driving force regulating phytoplankton community composition and nutrient cycling in the oceans [1, 2]. Yet, little is known about mechanisms that influence viral dispersal in aquatic systems, other than physical processes, and that lead to the rapid demise of large-scale algal blooms in the oceans [3, 4]. Here, we show that copepods, abundant migrating crustaceans that graze on phytoplankton [5, 6], as well as other zooplankton can accumulate and mediate the transmission of viruses infecting *Emiliania huxleyi*, a bloom-forming coccolithophore that plays an important role in the carbon cycle [7, 8]. We detected by PCR that >80% of copepods collected during a North Atlantic *E. huxleyi* bloom carried *E. huxleyi* virus (*Eh*V) DNA. We demonstrated by isolating a new infectious *Eh*V strain from a copepod microbiome that these viruses are infectious. We further showed that *Eh*Vs can accumulate in high titers within zooplankton guts during feeding or can be adsorbed to their surface. Subsequently, *Eh*V can be dispersed by detachment or via viral-dense fecal pellets over a period of 1 day postfeeding on *Eh*V-infected algal cells, readily infecting new host populations. Intriguingly, the passage through zooplankton guts prolonged *Eh*V's half-life of infectivity by 35%, relative to free virions in seawater, potentially enhancing viral transmission. We propose that zooplankton, swimming through topographically adjacent phytoplankton micropatches and migrating daily over large areas across physically separated water masses [9–11], can serve as viral vectors, boosting host-virus contact rates and potentially accelerating the demise of large-scale phytoplankton blooms.**

## Results and Discussion

*Emiliania huxleyi* (Haptophyta) forms large-scale spring blooms, which exert a major influence on the global climate by increasing water albedo, the emission of sulfur volatiles to the atmosphere and the export of carbon to the deep oceans [7, 12–14]. Consequently, *E. huxleyi* is a key phytoplankton species for current studies on global biogeochemical cycles and climate modeling [7, 15, 16]. In recent years, it has become apparent that the turnover and fate of blooms is largely influenced by the activity of *E. huxleyi* virus (*Eh*V), a lytic, large double-stranded DNA coccolithovirus (family *Phycodnaviridae*) [17] that specifically infects and kills *E. huxleyi* cells [3, 4, 18]. However, as submicron size entities, viruses are constrained by low Reynolds number viscous forces, dispersing at slow diffusion coefficients ($D_v$), <20 $\mu m^2 \times s^{-1}$ [19]. In the case of the large *Eh*V virions (~180 nm [4]), we calculated the diffusion coefficients ranging from 1.75 $\mu m^2 \times s^{-1}$ to 2.08 $\mu m^2 \times s^{-1}$ in seawater at 10°C (typical during natural blooms [8]) and at 18°C (our laboratory conditions), respectively. Viruses can also be dispersed by advection that often entails little internal mixing in oceanic areas [20] and can create confined mesoscale water patches [21]. A scenario of slow rate of viral dispersal contrasts with the rapid viral-mediated decline of algal blooms that often cover thousands of square kilometers of the ocean's surface [22]. Therefore, elucidating the mechanisms that govern the dissemination of marine viruses over large distances is key for understanding viral-driven biogeochemical processes.

We used both field studies and laboratory-based experimentation to explore the hypothesis that zooplankton act as viral transmission vectors mediating the dispersal of viruses and accelerating the demise of *E. huxleyi* populations.

### Detection of *Eh*V from Copepods Collected in the North Atlantic

To test whether copepods can carry *Eh*V, we collected calanoid copepods (Figure 1A) during *E. huxleyi* blooms from two surface sites (NA-VICE 1 and NA-VICE 2) during an oceanographic cruise in the North Atlantic. The two sites resembled distinct bloom stages. NA-VICE 1 was characterized by low coccolithophore cell abundance (*E. huxleyi* cells per ml: $0.3 \times 10^3 \pm 0.1 \times 10^3$) but higher viral density (*Eh*V particles per ml: $9.4 \times 10^3 \pm 1.2 \times 10^3$), likely representing a late stage of infection, whereas NA-VICE 2 was characterized by higher coccolithophore concentration (*E. huxleyi* cells per ml: $1.2 \times 10^3 \pm 0.2 \times 10^3$) and lower viral density (*Eh*V particles per ml: $0.2 \times 10^3 \pm 0.1 \times 10^3$), probably representing an early stage of viral proliferation during an *E. huxleyi* bloom. DNA extracts from approximately 40 prewashed individual copepods from each site were screened by PCR for the presence of the *Eh*V major capsid protein gene (*MCP*). We found that in both sites, more than 80% of the copepods contained *Eh*V DNA (Table S1 available online). Further quantitative PCR (qPCR) analyses revealed viral abundances ranging from $1 \times 10^3$ *Eh*Vs per copepod to $25 \times 10^3$ *Eh*Vs per copepod, demonstrating the capacity of copepods to concentrate high viral titers, probably by ingestion of *Eh*V-infected cells. In contrast, less than 40% of these copepods contained *E. huxleyi* DNA (Table S1). This discrepancy between virus and algal loads per copepod may suggest that *Eh*Vs can be ingested as free virions or can be bound to cellular aggregates formed during cell lysis or even that

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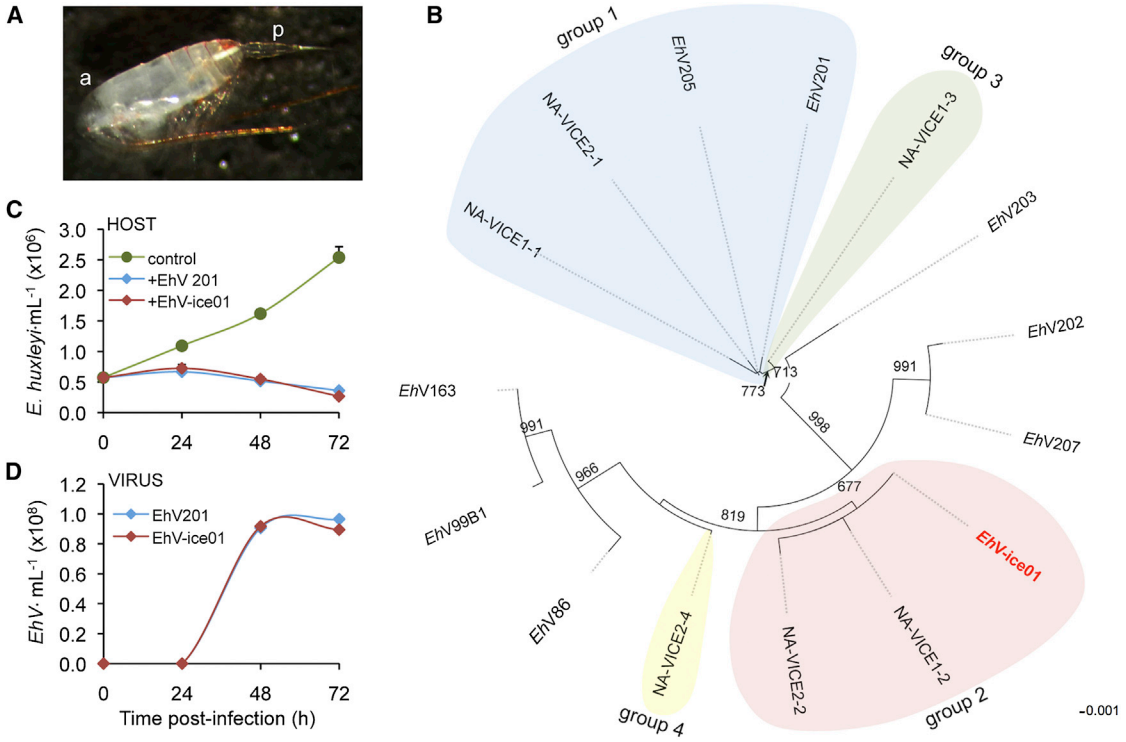


Figure 1. Diversity and Infectivity of *Eh*V Genotypes Isolated from Copepod Guts Collected in the North Atlantic

(A) Lateral view of a *Calanus* sp. copepod used in this study ("a" and "p" refer to the copepod's anterior and posterior sides, respectively).
(B) Neighbor-joining tree based on the *MCP* gene of *Eh*V genotypes amplified from individual copepods collected in the NA-VICE 1 and NA-VICE 2 sites. Distinct *Eh*V groups obtained from copepods are labeled from 1 to 4 and are encircled in different color tags.
(C and D) Dynamics of infection of *E. huxleyi* CCMP 374 by *Eh*V-ice01 (red) and *Eh*V 201 (blue), relative to noninfected control cells (green). *E. huxleyi* cell growth and *Eh*V production are shown in (C) and (D), respectively. The average and SD of three independent experiments are presented.

*Eh*V DNA may be more resistant to digestion in the copepod gut than algal DNA.

In order to assess the diversity of *Eh*Vs held within copepods, we sequenced the *MCP* amplicons obtained from copepod DNA extracts. We found that although collected in geographically separate locations, NA-VICE 1 and NA-VICE 2 shared similar sequence composition, with *Eh*V genotypes forming two main clusters, assigned here as groups 1 and 2 (Figure 1B). A few additional sequences were different, with sequences from NA-VICE 1 clustering only within group 3 and with others from NA-VICE 2 being present only in group 4 (representative sequences are presented in Figure 1B). Sequences from group 1 aligned with *MCP* sequences from the strains *Eh*V 201 and *Eh*V 205, previously isolated from the English Channel [4]. Sequences from groups 2, 3, and 4 formed unique clusters of *MCP* orthologs, thus representing new *Eh*V genotypes.

## Isolation of a New Infectious *Eh*V-Type Strain from Copepods

To test the viability of *Eh*V carried within copepods, we challenged *E. huxleyi* cultures with homogenates from copepods collected in the North Atlantic. We were able to isolate a new infectious *Eh*V-type strain using a copepod homogenate collected from a sediment trap placed at a depth of 50 m in the vicinity of NA-VICE 2. We coined this virus *Eh*V-ice 01, which distinctively branches within the novel *MCP*-group 2 (Figure 1B). This new viral strain displays a lytic mode of infection with an average burst size of ~300 viruses × cell$^{-1}$, comparable to those of other *Eh*Vs (Figures 1C and 1D). We further challenged multiple *E. huxleyi* strains for *Eh*V-ice 01 infectivity. Effective and reproducible lytic infections were obtained only on *E. huxleyi* CCMP 374 (Table S2).

## Mechanisms of Viral Acquisition and Dispersal by Zooplankton

Using a laboratory-based setup, we tested viral acquisition and dispersal by copepods through ingestion and defecation or physical attachment and detachment from the animal's surface.

To assess viral dispersal through ingestion and defecation, we fed freshly collected copepods with *Eh*V-infected *E. huxleyi* cells, transferred fecal pellets (FPs) produced over 24 hr to new *E. huxleyi* cultures, and assessed viral productivity over time (Figure 2A). qPCR analysis of each individual FP revealed an average of ~4,500 *Eh*Vs per copepod FP (Figure 2B). Transmission electron microscopy (TEM) analysis confirmed numerous intact electron-dense virions inside FPs, examined both after defecation and while still residing inside the copepod intestine (Figures 2C–2F). Subsequent treatment of *E. huxleyi* cultures with the viral-dense FPs resulted in the rapid viral-mediated lysis of the cultures, with concomitant *Eh*V production (Figures 2G and 2H). In contrast, control FPs derived from copepods fed with noninfected *E. huxleyi* cells did not impair host growth or result in viral production (Figures 2G and 2H). The efficiency of infection by *Eh*V within FPs had comparable dynamics to infection by free *Eh*V virions (Figures S1 and S2), indicating that FPs are potent vectors for *Eh*V transmission.

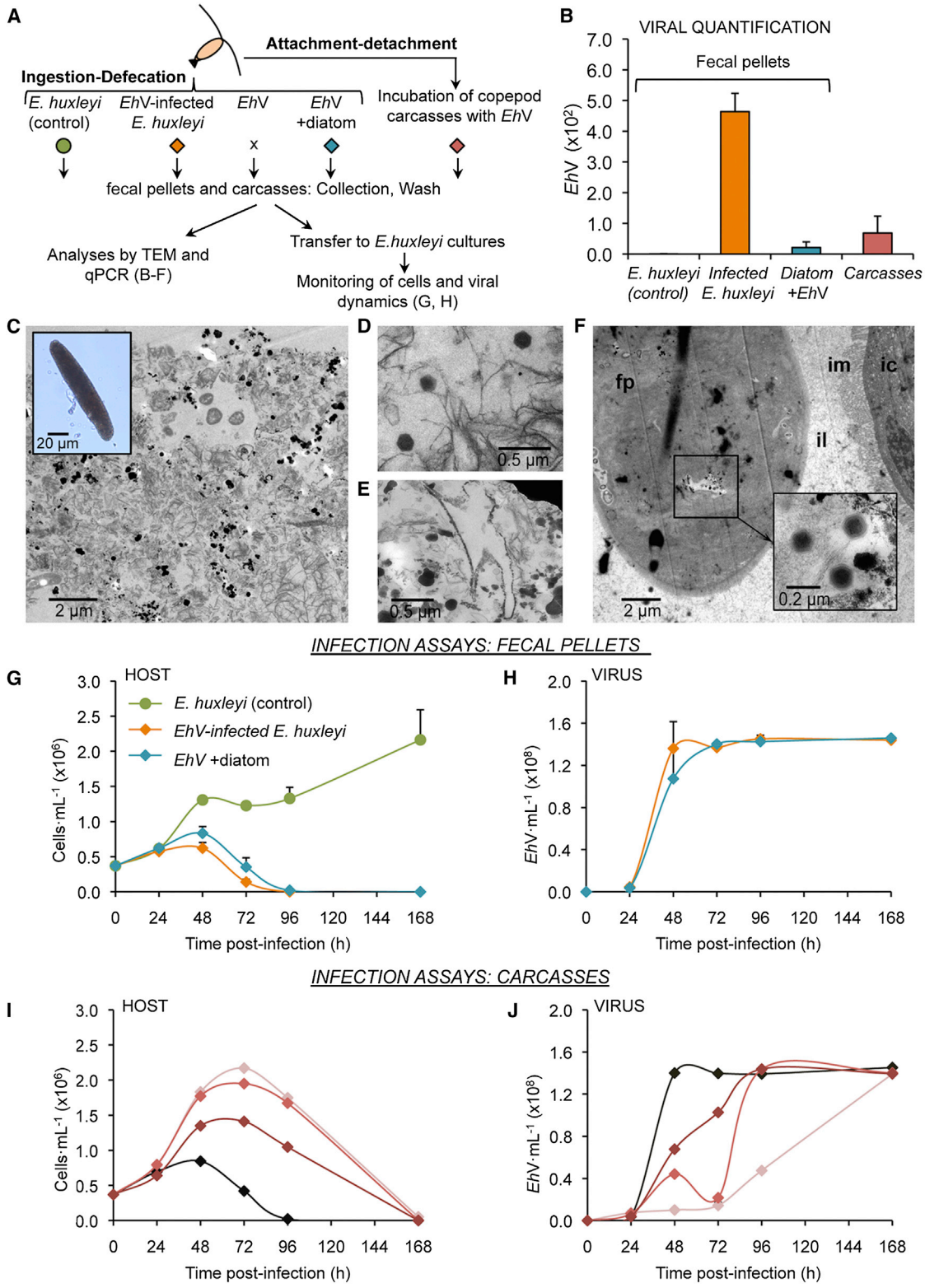


Figure 2. Dynamics of Infection by *Eh*V Derived from Copepod Fecal Pellets and Attached to Copepod Carcasses

(A) Scheme of experimental setup to examine viral acquisition and release by copepods.

(B) *Eh*V quantification in fecal pellets (FPs) collected 24 hr postfeeding on either *E. huxleyi* or *E. huxleyi* infected with *Eh*V or on the diatom *T. weissflogii* together with *Eh*V.

(C) TEM micrograph of a copepod FP. The inset image displays a copepod FP observed by light microscopy.

(D and E) TEM micrographs of *Eh*V particles and an *E. huxleyi* coccolith (Co) within an FP.

(F) TEM micrograph of an FP inside a copepod gut (ic, intestine cell; il, intestine lumen). The inset image zooms into an FP area region with *Eh*V particles.

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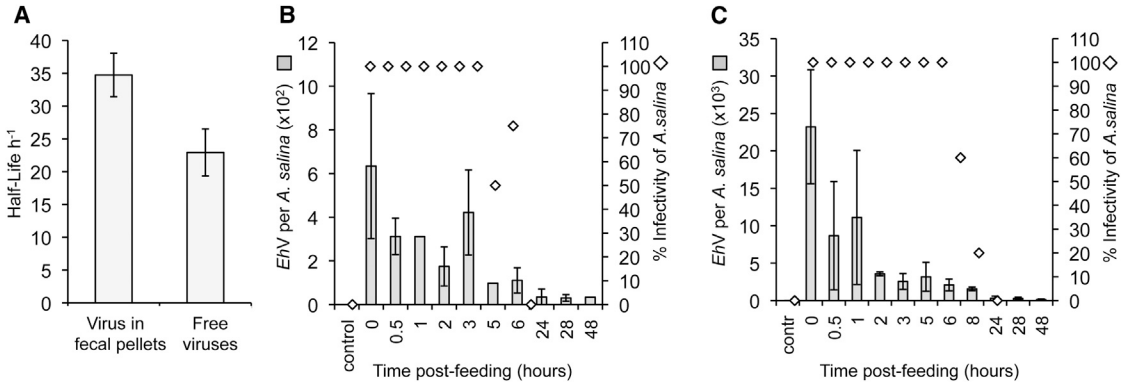


Figure 3. *Eh*V Decay Rates and Infectivity in Fecal Pellets, Grazers, and Free Virions

(A) *Eh*V decay rates within fecal pellets and within free virions in suspension (Student's t test, $p < 0.05$, $n = 3$; each biological replicate comprises six technical replicates per dilution and time point).

(B and C) *Eh*V infectivity of *A. salina* (nauplii stage, 3 days old in B and 5 days old in C, over 48 hr after feeding on *Eh*V-infected *E. huxleyi* cells). Bars represent the *Eh*V load per *A. salina* over time postfeeding, and diamonds represent the percentage of tested *E. huxleyi* cultures infected and killed by *Eh*V transmitted via *A. salina* over an indicated time. Average and SD of four independent experiments are presented.

We further found that *Eh*V can also be ingested and packed into FPs by copepods in the absence of infected host cells. When using *Eh*V as the sole food source, the copepods did not produce FPs. However, in the presence of both *Eh*V and the diatom *Thalassiosira weissflogii*, which is insensitive to this virus, the copepods produced FPs containing, on average, ~200 *Eh*Vs per FP (Figure 2B), which also remained highly infectious and killed new *E. huxleyi* cultures (Figures 2G and 2H).

To assess viral dispersal through attachment and detachment, we incubated carcasses of dead copepods with *Eh*V, transferred them to fresh *E. huxleyi* cultures, and monitored infection as performed for FPs. Analysis by qPCR revealed variable viral titers, ranging from ~100 to ~1,200 *Eh*Vs per carcass (Figure 2B), which consequently resulted in variable kinetics of infections (Figures 2I and 2J).

In order to expand our study to other zooplankters and complement our previous results, we performed additional experiments using the brine shrimp *Artemia salina* (nauplii stage) cultivated in the laboratory. We first tested the effect of ingestion and defecation on the viability of *Eh*V by determining the half-life of infective *Eh*Vs ingested and held within FPs relative to *Eh*V-free particles suspended in seawater (Figure 3A). We obtained a decay rate in infectivity for *Eh*Vs within FPs of 0.020 ± 0.002 $hr^{-1}$ and in suspended *Eh*V particles of 0.030 ± 0.006 $hr^{-1}$ (t test, $p < 0.05$, $n = 3$). This corresponded to average *Eh*V half-lives of 35 hr and 23 hr, respectively, and, thus, to decay rates 35% lower in *Eh*Vs residing within FPs.These data are in agreement with the TEM analysis that showed intact *Eh*Vs within the copepod gut and FP (Figures 2C–2F), suggesting that these viruses gain an advantage from passing through zooplankton. This resistance can stem from FP coating against light exposure and from dissolved enzymes or other chemicals, which can dramatically reduce viral infectivity [23, 24]. Our results therefore strongly suggest that zooplankton activity can significantly enhance *Eh*V transmission.

We further estimated the time frame in which *Eh*V can be dispersed following ingestion of infected cells. We found that zooplankton can disperse and serve as transmission vectors for over 1 day once fed with infected cells (Figures 3B and 3C). This result was obtained by transferring *A. salina* individuals previously fed with *Eh*V-infected *E. huxleyi* cells to new healthy *E. huxleyi* cultures over a period of 2 days. qPCR analyses showed that individual *A. salina* accumulated a variable range of *Eh*Vs, 100–30,000 viruses per zooplankton, similar to the natural viral load of copepods (Table S1), depending on their age and their size range (Figures 3B and 3C). After washing of remaining infected prey, 100% of the *A. salina* remained *Eh*V infective over 8 hr, and 20%–75% remained infective up to 28 hr, effectively transmitting *Eh*Vs that killed new *E. huxleyi* cells (Figures 3B and 3C). This result clearly suggests that zooplankton can retain viable *Eh*V virions for extended periods of time, allowing efficient transmission over relatively large spatial scales. Finally, we aimed to mimic the dispersal of infectious *Eh*Vs to healthy host populations via zooplankton vectors (Figure S3). We transferred three *A. salina* individuals previously fed with healthy (control) or *Eh*V-infected *E. huxleyi* cells to new *E. huxleyi* cultures, at initial densities similar to those found in natural blooms ($10^4$ cell × $mL^{-1}$ [8, 11]). The presence of control *A. salina* did not result in a detectable decline of *E. huxleyi* populations due to grazing. In contrast, in the presence of *A. salina* that carries *Eh*V, the host cultures readily lysed with concurrent viral production (Figure S3). We further compared the dynamics of *E. huxleyi* cultures following exposure to *Eh*V at low doses (viruses to host ratio or multiplicity of infection [moi] of 0.01) in the presence or absence of three *A. salina* individuals. In both cases, *E. huxleyi* cultures lysed; however, the decline in the presence of the grazer was considerably faster (Figure S3). We propose that the dynamic of infection can be considerably enhanced in the presence of grazers and will be most pronounced at the initiation phase of a bloom, where host-cell densities are low and effective contact rates are highly constrained.

(G and H) Dynamics of *E. huxleyi* infection initiated by adding FPs derived from the treatments described above (the color of the graph curves correspond to the types of FPs described in A). Average and SD of three independent experiments are presented.

(I and J) Dynamics of *E. huxleyi* infection using copepod carcasses previously exposed to *Eh*V. Different colors represent distinct single carcasses. Cellular and viral abundances were assessed by flow cytometry.

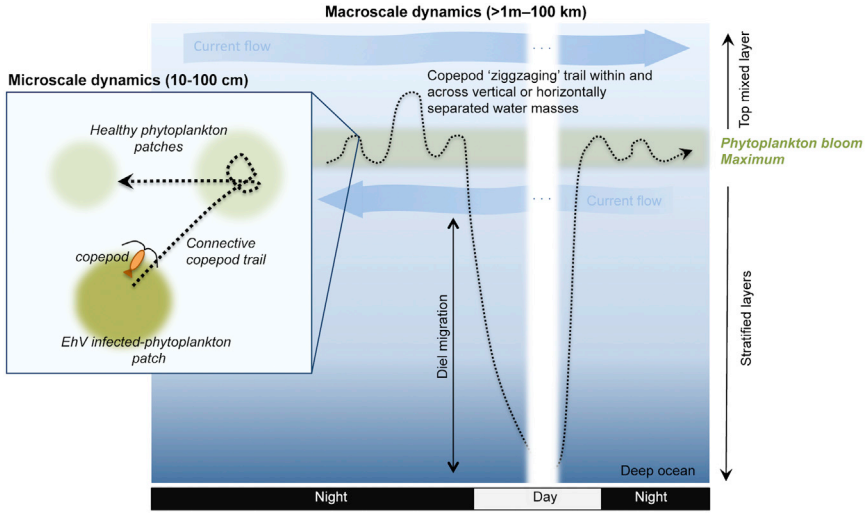


Figure 4. Microscale and Macroscale Modes of the Dispersal of Marine Viruses via Zooplankton Vectors

During phytoplankton blooms, migrating zooplankton disperse viruses within microscale patches by ingestion of infected algal cells or free viral particles attached to cell aggregates or by passive adsorption of viruses to the external cuticle. Viruses can then be transported between microscale patches within minutes through seascape topography, propagating local infection. At the macroscale, diurnal migrations across vertical water masses and through the pycnocline and stratified water concurrent masses can transport viruses to areas impassable to them and phytoplankton cells and can encompass large oceanic distances, creating a local hotspot of high viral density and increasing host-virus contact rates in remote, noninfected areas of the bloom.

## Zooplankton as Vector of Transmission for Algal Viruses

As illustrated in Figure 4, we propose that zooplankton that swim at speeds of tens of millimeters per second [9, 10] can rapidly connect viral-infected and noninfected phytoplankton centimeter-scale patches that characteristically compose the seascape topography [25–27]. This behavior, likely mediated by tracking the scent trail of diffused infochemicals from food particles and algal patches [10, 28], enhances the local propagation of viruses between neighboring patches within a given body of water, either via topical transport or ingestion-defecation cycles. *Eh*Vs can potentially be dispersed by diverse zooplankton species that are abundant during blooms [5, 29]. In our study, we show that *Eh*V can also hitchhike on large grazers, extending the conveyor-belt hypothesis [30] to oceanic viruses. Additionally, we provide strong evidence that *Eh*V can be transmitted not only via attachment and detachment but also effectively via ingestion and defecation. As we estimated, the diffusion coefficient of *Eh*V is $\leq 2\ \mu m^2 \times s^{-1}$, which lies at the lower end of previous estimates for viruses diffusing in liquid medium [19]. These slow rates of *Eh*V dispersal can thus be highly promoted by the activity of copepods, carrying and depositing infectious particles (with lower decay rates; Figure 3A), within host patches along their feeding path. These focal points can enhance host-virus contact rates and dramatically reduce the threshold dependency on host-cell densities to prompt effective viral-induced bloom demise. Moreover, given the fact that zooplankton can retain infectious viruses up to 28 hr after interacting with infected cells (Figures 3B and 3C), we propose that zooplankters can translocate viruses over significantly large distances. Copepods and other grazers can actively migrate up and down the water column over tens to hundreds of meters on a daily basis [31–33] (often explained as a strategy to avoid predation by visual hunters during the day [34]), piercing through stratified waters and maintaining their position near convergent fronts and pycnoclines, where current flows are reduced and food is abundant [11, 35, 36]. Interestingly, *E. huxleyi* blooms typically form a peak layer just above the pycnocline boundary layer [8, 37]. Copepods also perform short migrations between feeding zones with frequencies on the order of minutes to hours [38–40]. This long-range dynamic of copepods has the ability to connect counterflowing water masses hindered by vertical (physical) transport barriers, impenetrable to viruses and most phytoplankton groups. Therefore, at the macroscale (>1 km), transport by "zigzagging" copepods can drive viruses hundreds of meters or even kilometers away, where they can be exposed to new noninfected *E. huxleyi* populations and can prompt bloom demise over large oceanic scales.

In terrestrial ecosystems, the existence of vectors that transmit viruses to new hosts over large temporal or spatial scales is widely common [41, 42]. In aquatic systems, the understanding of these processes is still elusive. We provide evidence for the role of zooplankton-driven mechanisms that can enhance viral propagation in aquatic ecosystems, accelerating the turnover of marine phytoplankton biomass and nutrient recycling in the oceans, a mechanism that can be likely generalized to other microbial groups at sea. The interplay between the two most important top-down regulators (grazers and viruses) of the same prey (phytoplankton) adds a new dimension to the complex trophic interactions established during algal blooms and will have important implications for our understanding of host-pathogen coevolution and mechanisms of pathogen transmission among planktonic organisms.

### Accession Numbers

The GenBank accession number for the MCP sequence of EhV-ice01 reported in this paper is KF495209. The GenBank accession numbers for the other viral MCP sequences reported in this paper are KF495208, KF495210, KF495211, KF495212, KF495213, and KF495214.

### Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, three figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2014.09.031.

### Author Contributions

M.J.F. and A.V. conceptualized the project and wrote the manuscript. M.J.F., A.V., and V.F. collected the samples and performed the experiments. D.S. and I.K. participated in the TEM preparation, and S.B.-D. performed the phylogenetic analysis. J.E.O. provided the sediment trap samples. M.J.F., A.V., D.S., and I.K. analyzed the overall data.

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